Seeds

FIELD OF THE INVENTION

The present invention relates particularly, though not exclusively, to methods for modifying characteristics such as seed size in plants, especially flowering plants, and to plants and reproducible plant material produced by the methods. The invention also relates to nucleic acid constructs for use in such methods, as well as to modified plants and reproducible plant material per se.

BACKGROUND OF THE INVENTION

The seeds industry can be split into two high-value, commercial sectors: seeds for field crops such as corn, oil seeds, sugar beet and cereals, and vegetable and flower seed. The scientific improvement of crop plants has gone through a succession of innovations leading to the development of hybrid varieties for many crops and, most recently, to the introduction of genetically enhanced crops. The worldwide commercial seeds market is valued at around $30 billion (International Seed Federation).

1. Importance of seed size

Yield in crop plants where seed is the harvested product is usually defined as weight of seed harvested per unit area (Duvick, 1992). Consequently, individual seed weight is regarded as a major determinant of yield. Increasing seed size is desirable because it may increase total yield (Reynolds et al., 2001). There is also evidence that seed size (weight) is positively correlated with a number of components of 'seed quality' such as the percentage of germination (Schaal, 1980; Alexander and Wulff, 1985; Guberac et al, 1998); time to emergence (Winn, 1985; Wulff, 1986); durability (survival under adverse growing conditions) (Krannitz et al, 1991; Manga and Yadav, 1995); and growth rate (Marshall, 1986). Seed quality is an important factor in the cost of production of commercial seed lots since these must be tested before sale. Consequently, increasing total seed weight, even without increases in total seed yield, may have economic benefits through improvements in seed quality. Conversely, decreasing seed size may also be desirable in some circumstances, for example by facilitating water uptake required for germination (Harper et al., 1970), or in plants grown for their fruit.

Modification of seed size is also likely to improve yield through increasing the 'sink strength' of the seed (i.e. its capacity to demand nutrients from the seed parent), or increasing the period in which the seed is acting as a strong sink. It is well established that the demands of sink organs such as seeds have significant control over the rate of photosynthesis and the movement of photoassimilates from source to sink tissues (Patrick and Offler, 1995; Paul and Foyer, 2001). In wheat, the seed parent can supply more nutrients than developing seeds are able to demand for the first 15–20 days after pollination (Austin, 1980). Therefore modifications that enable seeds to draw nutrients earlier in development, for example by speeding up seed growth, will allow seeds to capture resources that would otherwise be wasted. An 'improved source-sink balance permitting higher sink demand during grainfilling' has also been proposed as a method for increasing yield in wheat (Reynolds *et al.*, 2001).

2. Composition of seeds

Mature seeds of flowering plants consist of three components: the seed coat, which is of exclusively maternal origin; and the two fertilization products, embryo and endosperm, which have maternal and paternal genetic contributions. Seeds develop from fertilized ovules. Ovule development has been described for many species (Bouman, 1984), including *Arabidopsis thaliana* (Robinson-Beers *et al.*, 1992; Schneitz *et al.*, 1995). The main structures of the mature ovule are: the embryo sac, which contains the female reproductive cells (egg and central cell); the nucellus, which surrounds the embryo sac at least partially; and the inner and outer integuments, which envelop the embryo sac and nucellus. After fertilization the embryo and nutritive endosperm develop inside the embryo sac while the integuments differentiate into the seed coat, which expands to accommodate the growing endosperm and embryo.

Most monocotyledonous plants, e.g. cereals including maize, wheat, rice, and barley (see Esau, 1965), produce albuminous seeds—that is, at maturity they contain a small embryo and a relatively massive endosperm. Most dicotyledonous plants, e.g. *Brassica napus,* (oil seed rape, canola), soybean, peanut, *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean) *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer aeietinum* (chick pea), and *Lens culinaris* (lentil), produce exalbuminous seeds—that is, the mature seeds lack an

endosperm. In such seeds the embryo is large and generally fills most of the volume of the seed, and accounts for almost the entire weight of the seed. In exalbuminous seeds the endosperm is ephemeral in nature and reaches maturity when the embryo is small and highly immature (usually heart/torpedo stage). Commonly embryo development depends on the presence of the endosperm, which is generally accepted to act as a source of nutrition for the embryo.

3. Control of seed size

Seed size control can be viewed from the perspective of (1) 'development' - the extent of cell division and expansion in one or more seed components (e.g. Reddy and Daynard, 1983; Swank *et al,* 1987; Scott *et al.*, 1998; Garcia *et al.*, 2003) or (2) 'metabolism'- metabolic activity and transport of nutrients within the seed and between the seed and seed parent (e.g. Weber *et al.*, 1996, 1997). Development and metabolism are interdependent: for example, invertase activity (involved in hexose transport) at the boundary of maternal tissues and endosperm or embryo sac is required for endosperm proliferation in maize (Cheng *et al.*, 1996), and high invertase activity is correlated with increased cell numbers in broad bean seed coat (Weber *et al.*, 1996), legume embryos (Weber *et al.*, 1997), and barley endosperm (Weschke *et al.*, 2003). Our present investigations focus on the developmental aspects of seed size control, although it can be assumed that changes to cell division/expansion in the seed will also be correlated with changes in metabolic activity and nutrient flow.

3a. Endosperm-led seed growth

Several studies show a correlation between endosperm growth and final seed size, for example in maize (Lin, 1984; Jones *et al.*, 1996), and even in the dicot *Arabidopsis thaliana*, which has an ephemeral endosperm (Scott *et al.*, 1998; Garcia *et al.*, 2003). Work in our laboratory has shown that overproliferation of the endosperm leads to large seeds with large embryos, while inhibition of endosperm proliferation produces small seeds with small embryos. We have manipulated endosperm proliferation and seed size using a variety of methods, including modifications to the ratio of paternally to maternally inherited chromosomes in the endosperm, cytosine methylation status of the parents contributing to the seed, and use of the *fis3/fie* mutation (Scott *et al.*, 1998; Adams *et al.*, 2000; Vinkenoog *et al.*, 2000). In these experiments we considered the resultant changes to seed growth to be

'endosperm-led', and effects on the embryo and the seed coat to be indirect. Some of our experiments specifically ruled out a direct effect on seed coat growth because the seed parent was wild-type and only the fertilization products were directly modified: for example, in the case of wild-type diploid seed parents crossed with tetraploid pollen parents, which produce large seeds (Scott *et al.*, 1998), or wild-type seed parents crossed with pollen parents hypomethylated by a *DNA METHYLTRANSFERASE 1* antisense construct, which produce small seeds (Adams *et al.*, 2000). Similarly, Garcia *et al.* (2003) described the *haiku* mutants of *Arabidopsis thaliana*, which produce small seeds due to early arrest of endosperm proliferation. The authors also noted a failure of cell elongation after fertilization in the integuments of *haiku* mutants, and concluded this was an indirect effect of limited endosperm growth.

3b Role of integuments/seed coat in establishing seed size

Alonso-Blanco *et al.* (1999) investigated seed size in wild-type plants of two *Arabidopsis thaliana* accessions, Cvi and Ler: seeds of the former weigh 80% more than seeds of the latter and are 20% longer. In both accessions, the authors found that 'seed coat and endosperm growth preceded embryo growth, determining the overall final length of the embryo and the seed'. They did find that the outer layer of the mature seed coat has more cells in Cvi than Ler, but did not investigate or comment on whether these extra cells were formed before or after fertilization. Moreover, the authors' inspection of mature unfertilized ovules showed that ovules in Ler were slightly longer than in Cvi, and therefore the authors concluded that 'ovule size differences could not account for the final Ler/Cvi seed size variation'. Their overall major conclusion was that 'the larger size of Cvi seeds compared with Ler is mainly because of the faster and prolonged growth of the integuments and the endosperm' (i.e. after fertilization); they did not address the question of whether this growth was led by the integuments or the endosperm. The authors suggested that the final cell number and size in the seed coat 'may be determined during ovule development', but significantly, there was no suggestion that a larger number of integument cells before fertilization was responsible for a larger final seed size.

Garcia *et al.* (2005) examined crosses between *Arabidopsis* mutant or transgenic plants that produce small seeds because of the inhibition of either endosperm growth or integument/seed coat growth. These authors proposed a model in which seed size is

determined by a reciprocal interaction between endosperm growth and elongation of integument/seed coat cells. They also reported that genotypes with fewer cells in the integument compensate by increasing cell elongation. The authors concluded, 'The final cell number in the integument [seed coat] is balanced by cell elongation and does not influence the size of the seed.'

Jofuku *et al.* (2005) and Ohto *et al.* (2005) reported that mutations in the *APETALA2* (*AP2*) gene increase seed size; Jofuku *et al.* (2005) also found that suppression of *AP2* activity through antisense or sense cosuppression had the same effect. *ap2* mutant seeds have seed coat abnormalities including large and irregular outer integument cells, lack of mucilage, and hypersensitivity to bleach; and the increase in seed size was found to be a mainly (Jofuku *et al.* , 2005) or wholly (Ohto *et al.*, 2005) maternal effect. However, neither paper investigated any possible correlation between (1) seed size and (2) cell number or any other aspect of integument/seed coat morphology in *ap2* mutants or transgenics.

Weber *et al.* (1996) compared growth of the seed coat in large- and small-seeded genotypes of *Vicia faba* (broad bean). They found that large-seeded genotypes contained more cells in the seed coat at 9 days after pollination, but cell numbers in the two genotypes were similar at 4 days after pollination. Therefore the number of cells in the integuments before fertilization could not be a factor in final seed size.

4. Relevant patent publications

(i) Fischer and Mizukami (2003), 'Methods for altering organ mass in plants', US Patent Application 20030159180

Mutations in the *AINTEGUMENTA (ANT)* gene of *Arabidopsis thaliana* prevent formation of the integuments (Klucher *et al.*, 1996; Baker *et al.*, 1997). Mizukami and Fischer (2000) describe the phenotype of *Arabidopsis thaliana* plants over-expressing the wild-type *ANT* gene under the control of the constitutive 35S promoter. Ectopic *ANT* expression increases the size of many plant organs including seeds, as well as causing male sterility through failure of anther dehiscence. Most of the transgenic plants are also female sterile 'because of abnormally extended proliferation of the chalazal nucellar cells'. However weak

overexpressers could generate seeds after hand-pollination with wild-type pollen. 'The enlarged *35S::ANT* fruit included T2 seeds that were larger than normal (not shown in the application), because of enlarged embryos.' The size of unpollinated ovules, and the number or size of cells in the integuments/seed coat, were not investigated or discussed. The large seed size of *35S::ANT* seeds was attributed only to size of the nucellus and embryo. US patent application no. 20030159180 describes uses of a modified ANT polypeptide for altering the size of plant organs including seeds. It was reported that the transgenic plants had varying degrees of fertility that were not correlated with organ size. There was no investigation of the effect of expressing the modified ANT polypeptide on integument or seed coat growth.

(ii) Jofuku and Okamuro (2001), 'Methods for improving seeds', US patent 6,329,567

Mutations in the *APETALA2 (AP2)* gene increase seed size (Okamuro and Jofuku, 1997). The mutations have a maternal effect on seed size but the only phenotype described for the integument/seed coat in *ap2* mutants is that the cells of the outer layer of the seed coat are enlarged with an irregular shape, along with some other morphological abnormalities (Jofuku *et al.*, 1994). US patent no. 6,329,567 describes methods of modulating seed mass using *AP2* transgenes, but this patent does not assess any effect of the transgenes on the integuments or seed coat.

(iii) Lepiniec *et al.* (2003), 'Regulating nucleic acid for expressing a polynucleotide of interest specifically in the endothelium of a plant seed and uses thereof', WO 03/012106 A2

The *BANYULS (BAN)* gene is expressed exclusively in the inner layer of the inner integument (this layer is also called the endothelium) in early seed development (pre-globular stage) (Devic *et al.*, 1999). International patent application WO 03/012106 A2 describes use of the *BAN* promoter to drive expression of various genes specifically in the testa (the seed coat layer derived from the inner integument). The authors propose uses such as modifying the tannin or fibre composition, or the hormonal equilibrium, but no relevant expression cassettes were reported or described. Modification of seed size is also proposed but only in the context of reducing or ablating seeds in fruit crops. A *BAN* promoter::*BARNASE* construct was shown to ablate the endothelium.

(iv) Zinselmeier *et al.* (2000), 'Regulated expression of genes in plant seeds', WO00/63401

This patent application relates to expression of genes such as *ipt* that 'affect metabolically effective levels of cytokinins in plant seeds, as well as in the maternal tissue from which such seeds arise, including developing ears, female inflorescences, ovaries, female florets, aleurone, pedicel, and pedicel-forming regions', and to transgenic plants with enhanced levels of cytokinin that exhibit 'improved seed size, decreased tip kernel abortion, increased seed set during unfavorable environmental conditions, and stability of yield'. A nucellus promoter (nucellus is the maternal tissue surrounding the embryo sac and enclosed within the integuments) is among those suggested for driving expression cassettes, but integuments are not specifically mentioned in the patent application, nor were any maternal tissue-specific expression cassettes described. The disclosure of this patent application is particularly concerned with maize.

(v) Scott (2002), 'Modified plants',. WO/0109299

This patent application relates to methods for controlling endosperm size and development through use of an antisense *DNA METHYLTRANSFERASE 1* gene that reduces cytosine methylation. As described in WO01/09299, and in Section 3a, above, modification to the cytosine methylation status of the seed or pollen parent alters seed size by altering the rate and extent of endosperm proliferation. Therefore the disclosure of this patent application relates exclusively to 'endosperm-led' seed growth.

In summary, documents in the prior art do not include an understanding that altering the size of integuments specifically through increasing the number of cells before fertilization could affect seed size after fertilization. A small number of published papers and patent applications touch on a possible relationship between seed coat size and seed size but do not make a link between (1) integument growth pre-fertilization and (2) final seed size.

5. Integument-led seed growth

We were surprised therefore to discover in our laboratory a mutant, termed the *mnt-1* mutant, that produces enlarged seeds through a primary effect on the integuments. Specifically, we observed that the seed cavity (i.e. the space within the post-fertilization embryo sac) is longer than normal giving the embryo more space to grow as a result of an

increase in cell number in the integument. This was particularly surprising in view of the earlier research mentioned in section 3a above which indicated that changes in seed growth were 'endosperm-led'. It was also surprising in view of the work of Alonso-Blanco *et al* (1999) mentioned in section 3b above which did not suggest that an increase in number of integuments cells led to an increase in seed size; and also in view of the work of Weber *et al* 1999 who found similar numbers of cells in the seed coat in small and large-seeded genotypes of broad bean soon after fertilization; and also in view of the work of Garcia *et al.* (2005) who claimed that 'The final cell number in the integument [seed coat] is balanced by cell elongation and does not influence the size of the seed'; and also in view of the work of Jofuku *et al.* (2005) and Ohto *et al.* (2005) who found a maternal effect of the *ap2* mutation on seed size but did not report a correlation between cell number in the integuments or in the seed coat and final seed size.

6. Increased stem diameter in *mnt-1* mutants

Increased stem diameter is also desirable in agriculture, as it may lead to an increase in plant biomass, which may in turn increase yield (Reynolds *et al.*, 2001). Increased stem diameter and biomass are also desirable in certain crops such as trees and vegetables. Thicker stems are also desirable because this trait increases resistance to lodging, a serious problem that reduces yields in crops including cereals (Zuber *et al.*, 1999), soybean (Board, 2001), and oilseed rape (Miliuviene *et al.*, 2004). A further aspect of the *mnt-1* mutant phenotype is increased diameter of the stems.

DEFINITIONS

The following non-limiting definitions of terms used in this specification are given by way of explanation.

"Function" when used in relation to a gene embraces both the operation of that gene at a molecular level as well as the downstream effects of expression of the gene which may result in phenotypic changes.

"Nucleic acid sequence": refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end, including

chromosomal DNA, plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

"Orthologues": refers to genes derived from a comon ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologues when their nucleotide sequences and/or their encoded protein sequences have a high percentage of sequence identity and/or similarity. Functions of orthologues are often highly conserved among species.

"Homologue": A gene (or protein) with a similar nucleotide (or amino acid) sequence to another gene (or protein) in the same or another species.

"Promoter": a region or sequence located upstream and/or downstream from the start of transcription involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

"Plant promoter" refers to a promoter capable of initiating transcription in plant cells.

"Operably linked" refers to a functional linkage between a promoter and a DNA sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence. Generally, "operably linked" means that the nucleic acid sequences being linked are contiguous, and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

"Plant": includes whole plants, plant parts, and plant propagative material including: shoot vegetative organs and/or structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (e.g., the mature ovary), seedlings, plant tissue (e.g., vascular tissue, ground tissue), cells (e.g., guard cells, egg cells, trichomes and the like), and their progeny.

"Plant cell": includes cells obtained from or found in seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes,

sporophytes, pollen, and microspores as well as whole plants. The term "plant cells" also includes modified cells, such as protoplasts, obtained from the aforementioned tissues.

"Wild type" in the context of a plant or plant material which has been modified in some way refers to a comparable plant which has not been modified in that way and grown or produced under similar conditions. For a given plant it may be the genotype or phenotype that is found in nature or in standard laboratory stock. References in this specification to relative changes in characteristics of plants or plant material are relative to wildtype.

SUMMARY OF THE INVENTION

According to one aspect, the invention provides a method of modifying cell proliferation in a plant which comprises modulating the expression of a gene whose expression or transcription product is capable of directly or indirectly modulating cell proliferation in the plant or plant propagating material, whereby cell proliferation , within the integuments and/or seed coats of the plant, is modified. Cell proliferation in other parts of the plant may also be modified. For example, in the stem of the plant.

According to another aspect, the present invention provides a method of modifying cell proliferation in a plant which comprises the step of transforming a plant or plant propagating material with a nucleic acid molecule comprising at least one regulatory sequence, typically a promoter sequence, capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified. Preferably, the overall size of the integuments/seed coat in the plant is modified. This may be useful where a product is produced in the integument/seed coat. In some embodiments, this will be achieved without affecting the growth or development of any part of the plant other than the seed.

In one embodiment, the function of a gene or gene product that promotes cell division is enhanced or the function of a gene or gene product that represses cell division is inhibited. Cell division in the integuments/seed coat may be increased resulting in a larger seed compared to wild type. Ths may be advantageous because increases in seed size can be achieved which are desirable as mentioned above. The seed may be at least 15%, or 25%, larger than wild type. More specifically, the seed may be at least 5% , 10%, 20%, 30%, 40%, 50%, 75%, 100%, 150%, or even 200% heavier than wild-type.The number of cells in the integuments/seed coat of the plant may be increased compared to wild type. The number of cells in the integuments/seed coat of the plant may be increased by at least 30%, or 50%, compared to wild type.

The diameter of the stem of the plant may be greater, for example at least 10% greater, than wild type. Preferably, the diameter of the stem of the plant is at least 20% greater than wild type. This may be advantageous as discussed above.

The sepal length of the plant may sufficiently greater than wild type to inhibit flower opening. For example, the sepal length may be at least 20% , or at least 50% greater than wild type.

Equally, the method allows the production of smaller seeds which can also be advantageous as mentioned above, and in another embodiment, the function of a gene or gene product that promotes cell division is inhibited or the function of a gene product that represses cell division is enhanced. Cell division in the integuments/seed coat may decreased resulting in a smaller seed compared to wild type. The seed may be at least 5% smaller than wild type, preferably 25% or more. The number of cells in the integuments/seed cost may be decreased compared to wild type. In particular the number of cells in the integuments/seed coat may be reduced by at least 30%, or 50%, compared to wild type.

The function of a gene that modulates cell proliferation may be enhanced compared to wildtype. Transcription of the gene is activated. Activation of transcription results in increased levels of mRNA and/or protein encoded by the gene. Typically, levels of mRNA may be increased by at least 20%. For example, the levels of mRNA may be increased by 50% or 75% or more.

A plant promoter may be operably linked to a coding region of the gene in the sense orientation. The function of the gene may be modulated by operably linking a plant promoter to a nucleic acid fragment from the gene to form a recombinant nucleic acid molecule such that an antisense strand of RNA will be transcribed.

The function of a gene may be modulated by introducing a nucleic acid fragment of the gene into an appropriate vector such that double-stranded RNA is transcribed where directed by an operably linked plant promoter. Decreased levels of mRNA and/or protein encoded by endogenous copies of the gene may be produced. Levels of mRNA and protein encoded by homologues of the gene may be reduced.

The function of the gene may be modulated by operably linking a plant promoter to a 'dominant negative' allele of the gene, which interferes with the function of the gene product.

The plant may be monocotyledonous, and is preferably a crop plant. For example, the plant may be *Tritcum spp* (wheat), *Oryza sativa* (rice), *Zea mays* (maize), *Hordeum spp.* (barley), *Secale cereale* (rye), *Sorghum bicolor* (sorghum), or *Pennisetum glaucum* (pearl millet). Alternatively the plant is dicotyledonous. For example, the plant is *Brassica napus* (oil seed rape, canola) or any other *Brassica* species used to produce oilseeds (e.g. *Brassica carinata*), *Glycine max* (soybean), *Arachis hypogaea* (peanut), *Helianthus annuus* (sunflower), *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea), *Lens culinaris* (lentil), or *Linum usitatissimum* (flax, linseed).

Integument and seed coat development is similar in all species examined in the family Brassicaceae (Bouman, 1975), to which *Arabidopsis thaliana* belongs. In *Brassica napus*, a crop plant closely related to *Arabidopsis thaliana*, the seed coat is also very similar in structure (Wan *et al.*, 2002). Therefore modifications that affect growth and development of integuments/seed coat in *Arabidopsis thaliana* should be directly applicable to members of the Brassicaceae, including *Brassica napus*.

The mature seeds of monocots such as cereals have a distinct structure. However cereal ovules have fundamental similarities with ovules of *Arabidopsis thaliana* and other dicots, also consisting of integuments enclosing a nucellus and embryo sac.) In rice, for example, the inner integument encloses the ovule before fertilization, and its growth precedes that of the endosperm and embryo, as in *Arabidopsis thaliana* (Lopez-Dee *et al.*, 1999). Therefore modification to growth of the integuments/seed coat may also be effective in altering overall seed growth in cereal crops. Specifically in rice a modification to growth of the inner integuments may be useful in modifying seed size.

It is notable that the *INO* gene, which in *Arabidopsis thaliana* is expressed in the outer integument and required for its growth (Villanueva *et al.*, 1999), has been identified in *Nymphaea alba* (water lily), where it is also expressed in the integuments (Yamada *et al.*, 2003). As the Nymphaeaceae are basal eudicots, which are ancestral to both dicots and monocots, this suggests that the sequence and expression patterns of at least some integument genes will be conserved even among distantly related groups of flowering plants.

The present invention is complementary to the invention disclosed in WO01/09299. Modifications to endosperm-led and integument-led seed growth could be combined for an even larger effect. In some situations integument-led seed growth alone may be preferable, as it only requires modification to the seed parent, while endosperm growth is determined both by maternal and paternal contributions.

Where the regulatory sequence is a promoter, the promoter sequence may be constitutive, directing gene expression in most or all cells of the plant. An example of a constitutive promoter that may be used in some embodiments of the invention is the 35S promoter, derived from the gene that encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. Alternatively, the promoter sequence may be specific, directing expression exclusively or primarily in one organ, tissue, or cell type of the plant. A variety of plant promoters can be used in the invention to direct expression exclusively or primarily in the integuments or seed coat. Suitable plant promoters include those obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells, such as Agrobacterium or Rhizobium. Some embodiments use promoters expressed in the pre-

fertilization integuments. These include but are not restricted to the promoters of the following genes: *INO* (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) and *BEL1* (Reiser *et al.*, 1995; At5g41410; accession no. NM_123506). Other embodiments use promoters expressed in the seed coat after fertilization. These include but are not restricted to the promoters of the following genes: *BAN* (Devic *et al.*, 1999; At1g61720, accession no. AF092912), *TT1* (Sagasser *et al.*, 2002), *TT2* (Nesi *et al.*, 2001; At5g35550; accession no. NM_122946), *TT8* (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509), *TT12* (Debeaujon *et al.*, 2001; At3g59030, accession no. AJ294464), and *TT16* (Nesi *et al.*, 2002; At5g23260; accession no. NM_203094). A flower-preferred promoter that may be used is the promoter of the *LFY* gene (Weigel *et al.*, 1992; At5g61850, accession no. NM_125579), which can be used to obtain desired flower-specific effects such as reductions in flower opening. Where a promoter is to be introduced into a plant, a promoter-containing nucleotide sequence of up to 2000 bp would typically be used.

The use of other regulatory sequences than a promoter to direct expression within the integuments and/or seed coat is contemplated. An example is an intron directing tissue-specific expression (see e.g. Deyholos and Sieburth, 2000).

There are a number of genes known or suspected to be involved in modulating cell proliferation, either directly or indirectly. Some embodiments of the invention use genes involved in hormone response, biosynthesis, translocation, or other aspects of hormone action. These include but are not restricted to *MNT* (described above), *IPT1* (Takei *et al.*, 2001; At1g68460, accession no. AB062607), and *ARGOS* (Hu *et al.*, 2003; At3g59900, accession no. AY305869). Other embodiments use core cell cycle genes (Vandepoele *et al.*, 2002). These include but are not restricted to *CYCD3;1* (formerly Cycδ3; Soni *et al.*, 1995; Vandepoele *et al.*, 2002; At4g34160, accession no. X83371) and *CYCB1;1* (formerly Cyc1aAt; Ferreira *et al.*, 1994; Vandepoele *et al.*, 2002; At4g37490, accession no. NM_119913). Other embodiments use transcription factors involved in regulation of the extent or rate of cell proliferation. These include but are not restricted to *ANT* (Klucher *et al.*, 1996; At4g37750, accession no. NM_119937).

An expression cassette may be used either to enhance or inhibit the function of a gene that modulates cell proliferation.

One method of enhancing function is to activate transcription of the gene, resulting in increased levels of mRNA and protein encoded by the gene. This is achieved by linking a plant promoter to the coding region of the gene (either with or without introns) in the sense orientation.

Partial or complete inhibition of gene function in order to achieve desirable characteristics in plants such as fertility may be achieved or "engineered" in several ways. One method, which uses 'antisense technology', is to link a plant promoter to a nucleic acid segment from the desired gene such that the antisense strand of RNA will be transcribed (see e.g. Branen et al., 2003; Choi et al., 2003). Another method, which uses 'RNAi technology', is to link a plant promoter to a nucleic acid segment from the desired gene and place the resulting recombinant nucleic acid into an appropriate vector such that double-stranded RNA is transcribed (Wang and Waterhouse, 2001). Both of these techniques may result in decreased levels of mRNA and protein encoded by the endogenous copies of the gene. For example, levels of mRNA may be reduced by at least 20%, preferably by at least 50% so as to achieve usefully large seeds without compromising fertility compared to wildtype. A nucleic acid fragment for antisense or RNAi technology may also be designed to decrease levels of mRNA and protein encoded by homologues or orthologues of the gene. A third method of inhibiting gene function is to link a plant promoter to a 'dominant negative' allele of the gene, which interferes negatively with the function of the gene product (see e.g. Hemerly et al., 1995; Nahm et al., 2003). In the case of inhibition of genes (e.g., by antisense, or the use of RNAi technology) it will be recognized that the inserted polynucleotide sequence need not be identical, but may be only "substantially identical" to a sequence of the gene from which it was derived. Inhibition of gene function may be achieved by reduction of expression of the gene through a feedback loop acting on that expression.

Alternatively, the nucleic acid sequence is a mutant form of an auxin response factor encoding gene, or a construct that inhibits expression or function of an auxin response factor. The auxin response factor gene may be MNT in the case of Arabidopsis thaliana or its orthologues in other species. For example, in the case of Brassica napus the gene may be BnARF2 as used in Example 2 below. In the case of rice the gene may be OsARF2. The mnt-1 mutant phenotype shows that the wild-type function of the MNT gene is to repress cell division in the integuments. Therefore inhibition of endogenous MNT expression or

function may result in larger integuments and a larger seed. Alternatively, enhancement of MNT expression or function may result in a smaller seed. In some situations, overexpressing the MNT gene may, in fact, result in a larger seed size possibly due to a feedback loop on the expression of the MNT gene.

In some embodiments of the invention, cell division in the integuments/seed coat will be increased, resulting in a larger seed compared to wild type. This may be achieved by enhancing function of a gene or gene product that promotes cell division, or inhibiting function of a gene or gene product that represses cell division. In other embodiments, cell division in the integuments/seed coat will be decreased, resulting in a smaller seed. This may be achieved by enhancing function of a gene or gene product that represses cell division, or inhibiting function of a gene or gene product that promotes cell division. In these embodiments the gene or gene product may be MNT or an orthologue of MNT; alternatively it may be another gene or gene product that affects cell division.

A plant may be further modified to maintain desirable characteristics may have been otherwise lost as a result of the transformation step. For example, the desirable characteristic may be fertility.

The plant may be engineered or bred further to maintain or introduce desirable characteristics. For example, the plant may be bred so that it is heterozygous for the modulated gene which directly or indirectly modifies cell proliferation. In particular, we have found that plants heterozygous for the mnt mutation have normal flowers and normal fertility, but that their seeds that are consistently significantly heavier than wild-type (typically about 10-20%), though not as heavy as seeds from mnt homozygous mutants. In other words, if MNT function is reduced by about 50% rather than abolished completely, the plants produce desirable heavier seeds without compromising fertility. For example, plants may be engineered as described above in order to reduce mRNA/protein levels for the cell proliferation gene.

For example, in further embodiments of the invention, MNT function is restored to petals and stamens of an mnt mutant such that seeds have the enlarged mnt-1 mutant phenotype but

fertility is not impaired. This may be achieved by operably linking the promoter of a gene that directs expression in petals and stamens but not carpels (which contain the ovules), such as AP3 (Jack et al., 1992), to the wild-type MNT gene. In different species, different wild type genes may be supplied. In other embodiments, MNT function may be restored to sepals and petals of an mnt mutant such that seeds have the enlarged mnt-1 mutant phenotype but fertility is not impaired. This may be achieved by operably linking the promoter of a gene that directs expression in sepals and petals but not carpels, such as AP1 (Mandel et al., 1992), to the wild-type MNT gene. In different species, different wild-type genes may be supplied.

According to another aspect of the invention there is provided a plant which includes a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified. The plant may have been obtained by a method in accordance with the invention and will have the resulting features in terms of genetic structures and phenotype as set out in any of claims 76 to 156 and/or described above.

According to a further aspect of the invention there is provided reproducible or propagatable plant material including a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within integuments and/or seed coat and at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby on expression of that nucleic acid sequence cell proliferation is modified.

According to another aspect of the invention, there is provided a method of modifying cell proliferation in a plant which comprises the step of modulating the response of the plant to an auxin whereby the overall cell number of the integuments/seed coat of the plant is modified. The response to an auxin may be modified by altering the expression of an auxin response factor. Preferably, the auxin response factor is ARF2. The function of a gene encoding the auxin response factor may be modulated so as to affect the function of the

factor. In the case of Arabidopsis thaliana, the gene may be MNT. In the case of Brassica napus the gene may be BnARF2. In the case of rice the gene may be OsARF2. Orthologues of these genes may be used in other species.

Most preferably, the function of an endogenous auxin response factor encoding gene is modulated for example by RNAi technology as described above. Most preferably, the function of that gene in the integuments/seed coat is affected.

In a further aspect of the invention, there is provided a method of modifying the function of a gene that directly or indirectly modulates cell proliferation, such as *MNT,* in a plant such that the seeds are enlarged but characteristics such as flower opening and/or fertility, preferably both flower opening and fertility, are not impaired. The seeds may be at least 10% or 20% larger than wild type. This may be achieved by breeding a plant that is heterozygous for a mutation in the gene such as *MNT* or an orthologue of that gene in other species. In another embodiment the plant has a partial loss-of-function mutation in a gene the function of which affects cell proliferation , such as *MNT* or an orthologue in other species. In another embodiment, the level of the RNA of that gene and protein is reduced in a wild-type plant by 30%, 40%, 50%, or 60%, for example by operably linking a promoter, such as the constitutive 35S promoter, to a nucleic acid fragment from the gene to form a recombinant nucleic acid molecule such that an antisense strand of RNA will be transcribed; or to nucleic acid fragments of the gene in an appropriate vector such that double-stranded RNA is transcribed.

According to a further aspect, the present invention provides a method of modifying cell proliferation in a plant which comprises the step of transforming a plant or plant propagating material with a nucleic acid molecule comprising at least one regulatory sequence, typically a promoter sequence, capable of directing expression within the stem of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified. Preferably, the overall size of the stems in the plant is modified. The stem may be at least 10%, 20%, 30%, or 40% greater in diameter than wild-type. In one embodiment of the invention, *MNT* function, or that of an orthologue, is at least partially inhibited in the stem, for example by operably linking a promoter such as the

constitutive 35S promoter to a nucleic acid fragment from the *MNT* gene or *MNT* orthologue to form a recombinant nucleic acid molecule such that an antisense strand of RNA will be transcribed; or to nucleic acid fragments of the *MNT* gene or *MNT* orthologue in an appropriate vector such that double-stranded RNA is transcribed.

In another embodiment of the invention, the function of a gene that directly or indirectly modulates cell proliferation such as *MNT* or an orthologue thereof is restored to flowers of an *mnt* mutant such that stems have the enlarged *mnt-1* mutant phenotype but fertility is not impaired. This may be achieved for example by operably linking the promoter of a gene that directs expression in flowers but not stems, such as *LEAFY* (*LFY*) (Weigel *et al.*, 1992; At5g61850, accession no. NM_125579), to the wild-type *MNT* gene. In different species, different wild type genes may be supplied.

Brief Description of the drawings

Embodiments in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figures 1 to 25 in which:

Figure 1A *Top:* Confocal micrographs of seeds with globular stage embryos from *mnt-1* (left) and wild-type (right) seed parents; *Bottom:* Mature seeds and embryos from *mnt-1* mutants and wild-type plants, photographed at the same scale;

Figure 1B is a scatter plot of number of seeds in each pod produced by *mnt-1* mutants vs mean seed weight in that pod, following controlled pollinations;

Figure 1C shows seeds from manually pollinated *mnt-1* and wild-type plants, and reciprocal crosses between them, photographed at the same scale;

Figure 2A shows light micrographs of mature unfertilized ovules, stage 3-VI (staging as in Schneitz *et al.*, 1995), from wild-type (left) and *mnt-1* (right) plants;

Figure 2B shows graphs showing number of cells, total length, and mean cell length for several integument layers in *mnt-1* and wild-type stage 3-VI ovules. (The width is also shown for layer ii1').

Figure 3 shows micrographs of the chalazal endosperm in developing seeds of *Arabidopsis thaliana*, all at the same scale. The *mnt-1* seed is an example of integument-led growth while the 2x X 6x seed provides an example of endosperm-led growth;

Figure 4A shows micrographs of *Arabidopsis thaliana* seeds illustrating endosperm-led seed growth illustrated by interploidy crosses in the C24 accession of *Arabidopsis thaliana* (see also Scott *et al.*, 1998);

Figure 4B shows micrographs illustrating integument-led seed growth illustrated by the *mnt-1* mutant in the Columbia accession of *Arabidopsis thaliana*;

Figure 4C is a micrograph of a seed illustrating the 'big bag' hypothesis;

Figure 5A-C is a photograph illustrating a comparison of floral phenotype and seed size in wild-type Col-3 (**5A**), *mnt-1* mutants (**5B**), and a Salk insertion mutant (Salk line 108995) homozygous for an insertion in the *ARF2* gene (**5C**);

Figure 5D is a photograph of a gel showing PCR-based scoring of segregants for the T-DNA insertion in Salk line 108995;

Figure 5E is a photograph of a gel showing scoring of presence of the insertion (top) and presence of homozygotes (bottom) in F1 progeny of the cross between an *mnt-1* homozygous mutant seed parent and the Salk 108995 homozygous pollen parent. All F1 progeny have a single copy of the insertion;

Figure 5F is a photograph illustrating floral and seed phenotype in an F1 hybrid plant resulting from a cross between a homozygous *mnt-1* mutant and a homozygous Salk insertion mutant (Salk line 108995);

Figure 6 is an alignment of wild-type *MNT* and mutant *mnt-1* cDNAs from translational start to stop;

Figure 7 is an alignment of wild-type MNT and mutant mnt-1 predicted proteins;

Figure 8 is an alignment of *Arabidopsis thaliana MNT* cDNA with its orthologue in *Brassica napus*, *BnARF2*;

Figure 9 is an alignment of *Arabidopsis thaliana* MNT predicted protein with its orthologues in *Brassica napus* (oilseed rape) (BnARF2) and *Oryza sativa* (rice) (OsARF2);

Figure 10 illustrates the BJ60, BJ40, pFGC5941, pART7, and BJ36 vectors used for the cloning strategies described in the following examples;

Figure 11 illustrates a cloning strategy for constructing reporter vectors (Example 3). In this and following figures, only restriction sites significant to the strategy are shown on the diagrams;

Figure 12 is a micrograph of a globular stage seed from a plant containing the *TT12::uidA* construct assayed for GUS expression; the inner layer of the inner integument is stained (arrow);

Figure 13 A illustrates a cloning strategy for constructing an RNAi vector to constitutively decrease *MNT* expression (Example 4);

Figure 13B is a series of photographs illustrating inflorescence and stem phenotypes (*top*) and seed sizes and weights (*bottom*) from independently transformed lines containing the 35S::MNT RNAi expression cassette compared with a wild-type control. Inflorescences and stems were photographed at the same scale, and seeds were photographed at the same scale;

Figure 14 illustrates a cloning strategy for constructing an RNAi vector to constitutively decrease *BnARF2* expression (Example 5);

Figure 15 illustrates a cloning strategy for constructing RNAi vectors to decrease *MNT* expression primarily in the integuments/seed coat (Example 6);

Figure 16 illustrates a cloning strategy for constructing RNAi vectors to decrease *BnARF2* expression primarily in the integuments/seed coat (Example 7);

Figure 17A illustrates a cloning strategy for constructing vectors for constitutive expression of *MNT* (Example 8) or *BnARF2* (Example 9);

Figure 17B is a series of photographs illustrating seed sizes and weights from independently transformed lines containing the 35S::MNT expression cassette compared with a wild-type control. Seeds were photographed at the same scale;

Figure 18 illustrates a cloning strategy for constructing vectors for expression of *MNT* in the integuments/seed coat (Example 10);

Figure 19 illustrates a cloning strategy for constructing vectors for expression of *BnARF2* in the integuments/seed coat (Example 11);

Figure 20 illustrates a cloning strategy for constructing vectors for expression of genes promoting cell division in the integuments/seed coat (Examples 12, 13);

Figure 21A is a series of photographs illustrating seed sizes and weights from individual primary transformants containing expression cassettes designed to increase seed size (*TT8::CYCD3;1* and *TT8::IPT1*) compared with controls (*TT8::uidA*). Data is taken from Table 2A. Seeds were photographed at the same scale;

Figure 21B is a series of photographs illustrating seed sizes and weights from transformed plants containing expression cassettes designed to increase seed size

compared with wild-type controls. Data is taken from Table 2B. Seeds were photographed at the same scale;

Figure 22 illustrates a cloning strategy for constructing a vector for expression of *MNT* in petals and stamens (Example 14);

Figure 23 illustrates a cloning strategy for constructing a vector for expression of *MNT* in sepals and petals (Example 15);

Figure 24A shows a wild-type Col-3 (*left*) and *mnt-1* (*right*) plant, illustrating the stem phenotype;

Figure 24B shows transverse sections of the inflorescence stem between nodes 2 and 3 as counted from the base of a wildtype from a wild-type (*top*) and *mnt-1* (*bottom*) plant. Each pair of images (low magnification, *left*; high magnification, *right*) was photographed at the same scale; and

Figure 25 illustrates a cloning strategy for constructing a vector for expression of *MNT* in flowers (Example 18).

DESCRIPTION OF PREFERRED EMBODIMENTS

Methods and products in accordance with the present invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The following vectors are used in the examples:

pGEMT (Promega, Southampton, UK)

BJ36, BJ40, BJ60 (gift of Bart Janssen, Horticultural & Food Research Institute of New Zealand)

pART7 (Gleave, 1992)

pFGC5941 (Cambia, Canberra, Australia; ChromDB, http://www.chromdb.org/plasmids)

Plant transformation protocols are based on Clough and Bent (1998) for *Arabidopsis thaliana* and Moloney *et al.* (1989) for Brassica.

Protein predictions and sequence alignments are carried out with GeneDoc software version 2.6.001 (Nicholas and Nicholas, 1997).

A. Identification of the *mnt-1* mutant

We identified the mutant, *megaintegumenta-1* (*mnt-1*), in a screen for large seeds yielded by a population of EMS (ethyl methanesulfonate)-mutagenized *Arabidopsis thaliana* in the Col-3_accession. Mature seeds produced by a seed parent homozygous for the *mnt-1* mutation are larger and more pointed than wild-type, with extra cells in the seed coat, and contain larger embryos (Fig. 1A). Specifically, Fig 1A shows that *mnt-1* mutants produce larger seeds with more cells in the seed coat (counts are for ii1, the outer layer of the inner integument).

Seeds collected from self-pollinated *mnt-1* mutant plants are up to twice the weight of wild-type Col-3 seeds (Table 1A).

Table 1A Seed weights in µg from *mnt-1* and w.t. Col-3 crosses, self seed, all siliques left on plant

	***mnt* self**	**w.t. self**
	27.1 (n=60)	14.4 (n=178)
	27.9 (106)	15.8 (75)
	29.7 (127)	
Mean	28.2	15.1
Range	27.1 to 29.7	14.4 to 15.8
Standard error	0.8	1.4

n = number of seeds weighed from each plant

However, *mnt-1* mutant plants are self-sterile until late in development due to floral abnormalities (see below), raising the possibility that the mutant produces large seeds because there are few seeds requiring maternal resources. Therefore we also conducted controlled pollinations in which only three siliques (seed pods) were allowed to set seed per plant, for both *mnt-1* and wild-type (Table 1B).

Table 1B Seed weights in µg from *mnt*-1 and w.t. Col-3 crosses, manual pollinations, 3 siliques per plant

	mnt* X *mnt	***mnt* X w.t.**	**w.t. X *mnt***	**w.t. X w.t.**
	31.1 (n=32)	30.4 (n=36)	30.5 (n=20)	30.6 (n=34)
	37.7 (37)	32.6 (22)	28.2 (30)	29.7 (34)
	31.4 (37)	37.4 (43)	28.2 (32)	22.1 (49)
	39 (20)	31.8 (17)	27.1 (46)	31.5 (31)
	33.7 (50)	34.4 (33)	26.5 (44)	29.6 (28)
	40.5 (22)	36 (50)	28 (44)	26.9 (53)
	38.3 (9)	37.1 (49)	30.3 (44)	30.2 (61)
	39.1 (11)	38 (24)		31.7 (48)
	37.2 (25)			21.2 (32)
	35.2 (24)			
	35.7 (43)			
	39 (31)			
	38.3 (64)			
	34 (23)			
	36.1 (33)			
	35.1 (54)			
Mean	36.3	34.7	28.4	28.7
Range	31.1 to 40.5	31.8 to 38.0	26.5 to 30.5	22.1 to 31.7
Standard error	0.7	1.0	0.6	1.3

n = number of seeds in silique

ttest [*mnt* X *mnt*] vs [w.t. X w.t.]: P < 0.000, significant

ttest [*mnt* X *mnt*] vs [*mnt* X w.t.]: P > 0.2, not significant

ttest [w.t. X w.t.] vs [w.t. X *mnt*]: P > 0.9, not significant

This treatment raised the mean weight of wild-type seeds by 90% and *mnt-1* seeds by 29%, indicating that low seed number is a component of large seed size in *mnt-1* mutants but that the *mnt-1* mutation also has a significant effect. On average *mnt-1* seeds weighed 26% more than wild-type when only three siliques per plant set seed; the difference in weights was significant at P<0.000. We also investigated whether occasional low seed set within

individual *mnt-1* siliques might raise seed weight; however a scatter plot (Fig. 1B) of mean seed weight of each pod vs number of seeds per pod following controlled pollinations in *mnt-1* (data from Table 1B) shows no correlation.

We also compared the weight of seeds produced by *mnt* heterozygotes with the weight of seeds produced by wild-type plants (Table 1C). We generated the heterozygotes through crosses in both directions, i.e. [w.t. X *mnt-1*] (designated [wXm]) and [*mnt-1* X w.t.] (designated [mXw]). We found that the weights of seeds from w.t. and *mnt-1* heterozygous plants were significantly different (t-test, H_0 w.t. = *mnt-1* heterozygous, P=0.0002).

Table 1C Seed weights in µg from w.t. Col-3 and *mnt-1* heterozygous plants, self seed, all siliques left on plant

	w.t.	***mnt* heterozygous**
Plant 1	17.1 (n=35)	19.4 (n=44) [wXm]
Plant 2	16.5 (37)	20.8 (60)
Plant 3	16.4 (49)	19.3 (50)
Plant 4	16.2 (50)	18.8 (50)
Plant 5	16.3 (45)	19.5 (46)
Plant 6	16.2 (40)	20.0 (62)
Plant 7	15.3 (52)	19.3 (44)
Plant 8	16.6 (42)	18.4 (59)
Plant 9	17.5 (54)	18.7 (45)
Plant 10	17.9 (56)	18.3 (57)
Plant 11		16.2 (64) [mXw]
Plant 12		17.7 (67)
Plant 13		20.0 (42)
Plant 14		17.3 (42)
Plant 15		18.3 (75)
Plant 16		17.1 (45)
Plant 17		17.0 (67)
Plant 18		17.7 (49)
Plant 19		18.3 (54)
Plant 20		16.6 (40)
Mean	**16.6 (460)**	**18.4 (1062)**
Range	**15.3 to 17.9**	**16.2 to 20.8**
Standard error	**0.2**	**0.3**

We also conducted two further experiments to compare the weights of seeds from (a) wild-type plants, (b) *mnt-1* homozygotes, and (c) *mnt-1* heterozygotes under conditions of restricted pollination. In the first experiment, six siliques on the primary shoot were pollinated and all other siliques on the primary shoot were removed; but all secondary shoots were allowed to set self-seed (Table 1D). In the second experiment, only six siliques on the primary shoot were pollinated and all other siliques on the plant were removed (Table 1E). In both experiments we carried out manual pollinations on six siliques per plant to enable young *mnt-1* homozygous mutant plants to set seed.

Table 1D Seed weights in µg from w.t. Col-3, *mnt-1* homozygous, and *mnt-1* heterozygous plants, 6 siliques pollinated on primary shoot, secondary shoots allowed to self-pollinate

	w.t.	***mnt* homozygous**	***mnt* heterozygous**
Plant 1	24.7 (n=289)	35.5 (178)	31.0 (n=237) [wXm]
Plant 2	24.6 (336)	34.5 (217)	28.7 (275)
Plant 3	24.4 (337)	36.9 (224)	29.8 (140)
Plant 4	23.9 (223)	37.8 (227)	30.7 (195)
Plant 5	25.8 (135)	37.4 (149)	29.3 (260)
Plant 6			29.2 (109) [mXw]
Plant 7			29.6 (307)
Plant 8			29.0 (198)
Plant 9			26.8 (346)
Plant 10			27.6 (341)
Mean seed weight	**24.7 (1320)**	**36.4 (995)**	**29.2 (2408)**
Range	**23.9 to 25.8**	**34.5 to 37.8**	**26.8 to 31.0**
Standard error	**0.3**	**0.6**	**1.3**

n = number of seeds weighed from each plant
t-test: H_0 w.t. = *mnt-1* homozygous, P <0.0000, difference is significant
t-test: H_0 w.t. = *mnt-1* heterozygous, P <0.0000, difference is significant

Table 1E Seed weights in µg from w.t. Col-3, *mnt-1* homozygous, and *mnt-1* heterozygous plants, 6 siliques pollinated on primary shoot, secondary shoots removed

	w.t.	***mnt* homozygous**	***mnt* heterozygous**
Plant 1	33.0 (n=307)	38.7 (n=274)	37.1 (n=53) [wXm]
Plant 2	32.2 (222)	37.5 (221)	35.1 (300)
Plant 3	35.1 (74)	41.1 (226)	37.7 (347)
Plant 4	35.0 (252)	41.0 (302)	35.6 (110)

Plant 5	34.8 (230)	38.5 (205)	38.1 (195)
Plant 6			40.9 (193) [mXw]
Plant 7			37.8 (245)
Plant 8			36.7 (280)
Plant 9			38.1 (210)
Plant 10			39.9 (222)
Mean seed weight	**34.0 (1085)**	**39.4 (1228)**	**37.7 (2155)**
Range	**23.2 to 35.1**	**37.5 to 41.1**	**35.1 to 40.9**
Standard error	**0.6**	**0.7**	**1.8**

n = number of seeds weighed from each plant

t-test: Ho w.t. = *mnt-1* homozygous, P = 0.0004, difference is significant
t-test: Ho w.t. = *mnt-1* heterozygous, P = 0.0013, difference is significant

mnt-1 homozygotes and *mnt-1* heterozygotes produced heavier seeds than wild-type plants in both experiments, and the difference in weight was significant in all cases at $P < 0.002$. When secondary shoots were allowed to set seed, seeds from *mnt-1* homozygotes were on average 47% heavier than seeds from wild-type plants, and seeds from *mnt-1* heterozygotes were on average 18% heavier than seeds from wild-type plants. When secondary shoots were removed, so that only six siliques set on each plant regardless of genotype, seeds from *mnt-1* homozygotes weighed 16% more than wild-type, and seeds from *mnt-1* heterozygotes weighed 11% more than wild-type.

The *mnt-1* mutation has a maternal effect on seed size. That is, an *mnt-1* homozygous mutant seed parent yields large seeds regardless of whether it is pollinated by an *mnt-1* or wild-type plant, while a wild-type parent yields normal seeds even if pollinated by an *mnt-1* plant (Fig. 1C). In Fig 1C seeds produced by *mnt-1* seed parents are shown on top and seeds from wild-type seed parents are below. H = fertilization products (embryo and endosperm) are heterozygous for the *mnt*-1 mutation. This shows that seed size in *mnt-1* mutants depends on the genotype of the seed parent, not the fertilization products. This is also shown by the lack of a significant difference between seed weights from [*mnt-1* X *mnt-1*] and [*mnt-1* X w.t] seeds, and between [w.t. X w.t.] and [w.t. X *mnt-1*] seeds (Table 1B).

The primary difference between *mnt-1* and wild-type seeds is that the mutant seeds contain more cells in the seed coat. Comparison of ovule development in *mnt-1* and wild-type plants shows that *mnt-1* ovules are of normal size and morphology until they are near maturity, at which time we observe that both the inner and outer

integuments of *mnt*-1 ovules are significantly longer than in wild-type, primarily due to a significantly greater number of cells (Fig. 2). In relation to the results depicted in Fig. 2, in *Arabidopsis thaliana* and other members of the Brassicacea most cell division and expansion occurs in the integuments on the abaxial side of the ovule (marked on wild-type ovule in Fig. 2A). Similarly, the nucellus in rice is enveloped by the abaxial inner integument (Lopez-Dee *et al.*, 1999). In Arabidopsis, ii1, ii1', and ii2 are the three cell layers of the inner integument and oi1 and oi2 are the two layers of the outer integument. The cells of layer ii1', which does not completely span the embryo sac, significantly expand in width after fertilization as part of seed growth (Beeckman *et al.*, 2000). *mnt-1* ovules have longer integuments with extra cells and in some cases an extra layer (arrow), as well as a larger seed cavity (Fig. 2A). C = the 'curving zone' of the abaxial outer integument (the region overlying ii1'; Beeckman *et al.*, 2000), M = the 'micropylar zone', regions delimited with black bars (Fig. 2A). Measurements shown in Fig. 2B were taken for the abaxial integuments only. Layers ii1', ii1, and the curving zone of oi2 are longer in *mnt-1* mutant ovules, almost exclusively due to greater cell number. Mean cell length is greater in the micropylar zone of *mnt-1* ovules but smaller or not significantly different in the oi2 curving zone and the other integument layers measured. There is no difference in mean width between *mnt-1* and wild-type cells of layer ii1'.

The peripheral endosperm in *mnt-1* mutant seeds also generates more nuclei than in wild-type seeds. The mean number of peripheral endosperm nuclei in *mnt-1* seeds at the heart stage is 1150, compared with 550 for a wild-type Col-3 seed at a comparable stage; see Scott *et al.* (1998) for a description of endosperm morphology and the counting method. However, we consider there are two crucial differences between *mnt-1* mutant seeds and large seeds that show endosperm-led growth. First, the chalazal region of the endosperm, which becomes greatly enlarged in endosperm-led seeds (e.g. seeds from interploidy crosses generating paternal excess, crosses where the DNA of the seed parent is hypomethylated, or *fis* mutants), is of roughly normal size in *mnt-1* mutants (although the pinched shape of the chalazal pole of mnt-1 seeds results in a longer and narrower chalazal endosperm) (Fig 3). We measured the maximum cross-sectional area of the chalazal cyst plus nodules at 6 DAP, a stage at which differences are apparent between wild-type and paternalized endosperms

(Scott et al., 1998). Mean areas were 2690 μm^2 (±s.e.m. 328) for wild-type seeds (n=4) and 2537 μm^2 (±416) for *mnt-1* seeds (n=5), and there was no significant difference between the mutant and wild-type endosperms (t-test, H_0 w.t. = *mnt-1*, P=0.79). Second, the size difference between *mnt-1* and wild-type seeds follows from differences existing before fertilization i.e. before the endosperm has been created. The overproliferation of peripheral endosperm may follow from the larger seed volume created by enlarged integuments/seed coat.

B.The 'big bag' hypothesis

We observe that seeds with enlarged endosperms and seeds with large seed coats have a feature in common: the seed cavity (i.e. the space within the post-fertilization embryo sac) is larger than normal, giving the embryo more space to grow (Fig. 4A, 4B). Specifically, endosperm-led seed growth is illustrated by interploidy crosses in the C24 accession of *Arabidopsis thaliana* (see also Scott *et al.*, 1998). As shown in Fig. 4A extra paternal genomes produce seeds with a large cavity (top left, 2x X 6x cross), and ultimately large seeds with large embryos (2x X 4x cross, bottom left). Conversely, extra maternal genomes generate seeds with small cavities (top right, 6x X 2x cross), and ultimately small seeds with small embryos (4x X 2x cross, bottom right). The control 2x X 2x cross is shown in the middle.

In contrast in integument-led seed growth as illustrated in Fig. 4B the seeds also have a large seed cavity (top left) compared with wild-type (top right). Mature seeds and embryos are compared below.

This leads to our 'big bag' hypothesis, which states that seed and ultimately embryo size is set by the size of the seed cavity, which may be controlled by several factors including extent of endosperm proliferation and extent of integument/seed coat proliferation (Fig. 4C).

It is well established in the literature that after fertilization in *Arabidopsis thaliana* there is no further division in the seed coat, and growth occurs only by cell expansion (Léon-Kloosterziel *et al.*, 1994; Beeckman *et al.*, 2000; Windsor *et al.*, 2000). Obviously seeds with large endosperms must also have large seed coats; however, in this case, the seed coat

grows by cell expansion after fertilization. In seeds where large seed coat is considered the primary cause of seed enlargement (integument-led seed growth), the integument/seed coat contains extra cells, as observed in *mnt*-1 mutants.

C. Further aspects of the *mnt-1* mutant phenotype

The *mnt-1* mutation affects floral morphology as well as seed size. Most flowers fail to open; this is associated with a deviation from the normal ratio of sepal to petal length, so that the petals are shorter than the sepals. Specifically, *mnt* mutant sepals are about 60% longer than wild-type. This deviation is mainly due to overgrowth of the sepals caused by extra cell division, although under some conditions the petals also fail to expand normally. This characteristic may be commercially useful in some crop species. A smaller increase in sepal length may be sufficient to prevent flower opening whilst allowing self-fertilization. Additionally, pollen is shed from the anthers on to the sides of the carpel rather than the stigma. This is associated with overgrowth of the gynoecium caused by extra cell division, although under some conditions the stamen filaments do not extend normally.

The floral phenotypes result in sterility of plants unless manual pollination is carried out (*mnt-1* homozygotes are female fertile, and the pollen that completes development is also fertile). However the last few flowers produced by *mnt-1* mutants appear wild-type and these are self-fertile.

Germination frequency of *mnt-1* seeds is normal, and the seedlings are vigorous.

mnt-1 mutants have thick inflorescence stems compared with wild-type plants (Fig. 24).A comparison of primary inflorescence stems shows that *mnt-1* stems have a 20% greater diameter than wild-type (mean diameters *mnt-1*, 1.59 mm ± s.e.m. 0.04; w.t. , 1.32 mm ± .06; n=6 for each). Transverse sections (Fig. 24B) show that cells are of normal size in *mnt-1* mutant stems but many more cells are formed.

D. Molecular characterization of the wild-type *MNT* gene and *mnt-1* mutant allele

i) Wild-type MNT sequence

We mapped the *MNT* locus to a 60.9 kb region of chromosome 5 that was annotated by The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) to contain 17 genes. T-DNA insertion lines with insertions in these genes generated by The Salk Institute Genome Analysis Laboratory (SIGnAL) (Alonso *et al.*, 2003) (http://signal.salk.edu) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (http://nasc.nott.ac.uk). Salk line no.108995 (NASC stock no. N608995), with an insertion in the coding region of the *AUXIN RESPONSE FACTOR 2 (ARF2)* gene, included a plant homozygous for the insertion with a similar phenotype to *mnt-1* mutants, including closed flowers and large seeds (Fig. 5A–C). Genotypic scoring of segregants from the Salk 108995 family, including one heterozygote and the homozygote, is shown in Fig. 5D. Specifically in Fig. 5D *Top:* Scoring for presence of an insertion in the *ARF2* gene. Primers used were 5' TGG TTC ACG TAG TGG GCC ATC G 3', and 5' GAG TGG GTG GAG TGT GTT TG 3'. Lanes M and O show presence of the insertion. *Bottom:* Scoring for homozygous insertion mutants. Primers used were 5'GAG TGG GTG GAG TGT GTT TG 3' and 5' AGT TGG TTT TCG TTT GAG CAT 3'. PCR conditions are set so that the gene will only amplify if there is no insertion: therefore PCR products will be amplified from DNA extracted from wild-type plants and also those hemizygous for the insertion, but not homozygous plants. Lane M shows no amplification, indicating this plant is homozygous for the insertion. An allelism test was conducted by crossing a seed parent homozygous for the *mnt-1* mutation with the Salk 108995 homozygote as pollen parent. F1 progeny were hemizygous for the insertion (Fig. 5E) and had the *mnt-1* mutant phenotype (Fig. 5F), confirming that *MNT* is the *ARF2* gene.

MNT/ARF2 will be referred to as *MNT* in the remainder of this document. The *MNT* gene = At5g62000, accession no. NM_125593. The genomic DNA for *MNT*, including the coding region plus 4371 bases of 5' and 525 bases of 3' flanking region, is shown in SEQ ID NO. 1. SEQ ID NO. 2 is the complete cDNA, and SEQ ID NO. 3, the predicted protein.

ARFs form part of the system for responding to auxin, a hormone known to be involved in many plant developmental processes including cell division and expansion (Stals and Inzé, 2001; Leyser, 2002). ARFs are transcription factors that in general are not induced by auxin

themselves but which regulate expression of auxin-inducible genes, such as members of the Aux/IAA class (Liscum and Reed, 2002). ARFs have been shown to bind to Auxin Response Elements (AREs) containing the motif TGTCTC in the promoters of auxin-inducible genes (Ulmasov *et al.*, 1999a). Twenty-two ARFs predicted to be functional have been annotated in the *Arabidopsis thaliana* genome (Hagen and Guilfoyle, 2002). ARFs contain two conserved domains—an N-terminal DNA binding domain and a C-terminal dimerization domain—and a variable middle region. An ARF may activate or repress transcription of its targets and this is thought to depend on the sequence of the middle region (Ulmasov *et al.*, 1999b). Evidence so far suggests that ARF2 is likely to be a repressor (Tiwari *et al.*, 2003).

ii) Mutant mnt-1 sequence

We sequenced the coding region from genomic DNA of the *mnt-1* allele plus 4371 bases of the 5' and 525 bases of the 3' flanking regions (this genomic sequence is shown in SEQ ID NO. 4). A single base change with respect to the wild-type Col-3 sequence, from G to A, was identified at position 665 from translational start, at the end of intron 3. This was predicted to affect splicing by changing the conserved 3' splice site (Brown and Simpson, 1998) from the consensus AG sequence to AA. We sequenced the first 837 bases of the *mnt-1* cDNA from start of translation and confirmed that four bases are deleted from the beginning of exon 4. The *mnt-1* cDNA from translational start to stop, consisting of the 837 directly sequenced bases plus the remainder of the cDNA coding region as predicted from the sequenced *mnt-1* genomic DNA, is shown in SEQ ID NO. 5. Wild-type *MNT* and mutant *mnt-1* cDNA sequences are aligned in Fig. 6.

The predicted mnt-1 protein (SEQ ID NO. 6) has a frameshift from amino acid position 123 and an early stop codon at position 167. Wild-type MNT and mutant mnt-1 predicted protein sequences are aligned in Fig. 7. The frameshift and early stop codon are both within the DNA binding domain and therefore the mnt-1 allele is likely to cause a complete loss of MNT function.

Example 1

Value of mnt mutants in understanding and modifying growth of integuments/seed coat.

The mnt mutant seed phenotype demonstrates that there is a correlation between the size of integuments before fertilization and the size of the mature seed in Arabidopsis thaliana (Figs 1, 2). Due to the similarities in seed structure among even distantly related groups of flowering plants, this leads to the expectation that modification to integument/seed coat size in other species, and certainly in members of the Brassicaceae such as Brassica napus, will also result in changes to seed size.

Our knowledge of the mnt mutant phenotype and MNT gene sequence can be exploited in other species through TILLING ('Targeting induced local lesions in genomes'). In this reverse genetics technique, chemically mutagenized populations are screened for presence of a point mutation in a nucleic acid sequence of interest; this can be done as a high-throughput procedure and is applicable to many species (Till et al., 2003). For example, TILLING could be applied to the Brassica napus or rice orthologues of MNT in order to modify seed size in these crop species.

Our knowledge that mnt-1 heterozygotes are self-fertile and produce larger seeds than wild-type plants shows that a plant with reduced MNT function (as in a heterozygote for an MNT mutation or in a plant which has been genetically modified in some way to achieve the same effect as conventional breeding) will advantageously produce large seeds without a loss of fertility.

Example 2 Modifying expression of MNT orthologues in other species.

Knowledge of the MNT sequence in Arabidopsis thaliana also allows us to search for orthologues in crop species as a necessary first step in targeted modification of the expression of the gene in these species.

By way of example, we amplified the putative Brassica napus orthologue (BnARF2) of MNT using primers (SEQ ID NO 7, 8) based on the MNT sequence and on publicly available Brassica oleracea sequence. The BnARF2 cDNA was amplified from total RNA isolated

from seedlings of Brassica napus var. Westar. The BnARF2 cDNA from translational start to stop is shown in SEQ ID NO. 9 and is aligned with Arabidopsis thaliana MNT cDNA in Fig. 8. The BnARF2 predicted protein (SEQ ID NO. 10) has 85% identity to Arabidopsis thaliana MNT.

A family of ARFs has also been characterized in rice and one of these, OsARF2 (accession no. AB071293), is considered to be the orthologue of Arabidopsis thaliana ARF2 (Sato et al., 2001). Fig. 9 shows an alignment of the predicted protein sequences of MNT (Arabidopsis thaliana ARF2), BnARF2, and OsARF2.

Orthologues of MMT may be determined for other species using similar techniques.

Example 3

Construction, transformation, and analysis of reporter vectors to show where integument/seed coat promoters are expressed in *Arabidopsis thaliana*.

This is to test which promoters are suitable for driving integument/seed coat-specific or -preferred expression of nucleic acids such as *MNT* antisense or RNAi constructs, or other genes modifying cell proliferation.

Diagrams of the BJ60, BJ40, pFGC5941, pART7, and BJ36 vectors used in the cloning strategies described in this and following examples are shown in Fig. 10.

The cloning strategy is shown in Fig.11.

3a Construction of reporter vectors

3a(i) *TT8*

A reporter vector based on the promoter of the *TT8* gene (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by the polymerase chain reaction (PCR) from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT8* gene using the primers TT8F and

TT8R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' AAACATATGCCAACGGGATCATGGGATTAC 3' **TT8F** SEQ ID NO. 11
NdeI
5' AAACTGCAGCGTTCCCGGAGATACGAAAAC 3' **TT8R** SEQ ID NO. 12
PstI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ60, 5' to the uidA reporter which includes a terminator signal, forming the vector TT8-BJ60.

3a(ii) *TT12*

A reporter vector based on the promoter of the *TT12* gene (Debeaujon *et al.*, 2000; At3g59030, accession no. AJ294464) is constructed as described below. A 1.7 kb fragment including the *TT12* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT12* gene using the primers TT12F and TT12R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *TT12* PCR fragment respectively.

5' AAACATATGGGAATTCACAATCGGAAAGTC 3' **TT12F** SEQ ID NO. 13
NdeI
5' AAACTGCAGGGTCCGTTTATTAGTTCCTC 3' **TT12R** SEQ ID NO. 14
PstI

The *TT12* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ60, 5' to the uidA reporter gene forming TT12-BJ60.

3b Construction of binary vectors and transformation into Arabidopsis thaliana

Reporter cassettes are excised with NotI from the following vectors:

TT8-BJ60
TT12-BJ60

and ligated into the NotI sites of the binary vector BJ40, forming the following vectors for transformation:

TT8-uidA-BJ40
TT12-uidA-BJ40

The binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

3c Analysis of expression patterns

The *uidA* gene encodes β-glucuronidase (GUS), which is assayed using standard protocols (e.g. Jefferson, 1987). For Figure 12 (below) the following assay was used. Seeds were dissected from siliques into GUS staining buffer (100mM Tris-HCl pH 7.2, 50 mM NaCl, 0.1% Triton-X-100, 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucoronic acid (X-Gluc), 2mM $K_3Fe(CN_6)$, 2 mM $K_4Fe(CN)_6$) and incubated overnight at 37° C.

Figure 12 shows a globular stage seed from a plant containing the *TT12::uidA* construct assayed for GUS expression; the inner layer of the inner integument is stained (arrow), indicating activity of the *TT12* promoter fragment in that integument.

Example 4

Construction and transformation of an RNAi cassette that decreases *MNT* expression in *Arabidopsis thaliana*, including decreased expression in the integuments/seed coat.

The cloning strategy is shown in Fig. 13A.

4a Construction of RNAi cassette

An RNAi vector based on the *MNT* gene (see above) is constructed as described below. A 0.57 kb fragment of the *MNT* cDNA ('*MNT*i') is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers FARF2i and RARF2inew which introduce XbaI and AscI sites at the 5' end of the *MNT*i PCR fragment, and BamHI and SwaI sites at the 3' end of the PCR fragment.

5' GATCTAGAGGCGCGCCGGATCTGAGAACTGGATG 3' **FARF2i** SEQ ID NO. 15

XbaI AscI

5' GAGGATCCATTTAAATCCGCAGCATCATTCAAGT 3' **RARF2inew** SEQ ID NO. 16

BamHI SwaI

The *MNT*i PCR fragment is A-tailed and ligated into pGEMT, and then excised with AscI and SwaI and ligated into the AscI and SwaI sites of the pFGC5941 RNAi vector 3' to the *35S* promoter and 5' to the *CHSA* intron, which places the fragment in forward orientation. This forms the vector 35S-MNTi-pFGC5941. The *MNT*i PCR fragment is then excised from pGEMT with BamHI and XbaI and ligated into the BamHI and XbaI sites of the 35S-MNTi-pFGC5941 vector, 3' to the CHSA intron and 5' to the *ocs* terminator signal, which places the fragment in inverse orientation. This forms the vector 35S-MNTi-inv MNTi-pFGC5941.

4b Transformation into Arabidopsis thaliana

Vector 35S-MNTi-inv MNTi-pFGC5941 is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

4c Analysis of seed weights in plants transformed with the 35S::MNT *RNAi vector*

Wild-type plants transformed with the *35S::MNT* RNAi vector described in Example 4a, b have the *mnt* mutant phenotype, including closed flowers for most of the plant's life cycle (Fig. 13B *top left*), inflorescence stems with increased diameter (Fig. 13B *top right*), and large seeds (Fig. 13B, *bottom*). Seeds from four independently transformed lines, along with wild-type plants grown under the same conditions, are shown in Fig. 13B (*bottom*). The

mean weight for these four lines was 35.3 μg, compared with 13.8 μg for the wild-type control.

Example 5

Construction and transformation of an RNAi cassette that decreases BnARF2 expression in *Brassica napus*, including decreased expression in the integuments/seed coat.

The cloning strategy is shown in Fig 14.

5a Construction of RNAi cassette

An RNAi vector based on the *BnARF2* gene (Example 2, above) is constructed as described below. A 0.56 kb fragment of the *BnARF2* cDNA (*BnARF2*i) is amplified by PCR from *Brassica napus* cDNA using the primers FBnARF2i and RBnARF2i which introduce XbaI and AscI sites at the 5' end of the *BnARF2*i PCR fragment, and BamHI and SwaI sites at the 3' end of the PCR fragment.

5'GATCTAGAGGCGCGCCGCGATATGAGAACTGGATA 3' **FBnARF2i** SEQ ID NO. 17

XbaI AscI

5'GAGGATCCATTTAAATGTAGGCCCCGCAGGGTCA 3' **RBnARF2i** SEQ ID NO. 18

BamHI SwaI

The *BnARF2*i PCR fragment is A-tailed and ligated into pGEMT and then excised with AscI and SwaI and ligated into the AscI and SwaI sites of the pFGC5941 RNAi vector 3' to the *35S* promoter and 5' to the *CHSA* intron using the enzymes AscI and SwaI, which places the fragment in forward orientation. This forms the vector 35S-BnARF2i-pFGC5941. The *BnARF2i* PCR fragment is then excised from pGEMT with BamHI and XbaI and ligated

into the BamHI and XbaI sites of the 35S-BnARF2i-pFGC5941 vector 3' to the CHSA intron and 5' to the *ocs* terminator, which places the fragment in inverse orientation. This forms the vector 35S-BnARF2i-inv BnARF2i-pFGC5941.

5b Transformation

Vector 35S-BnARF2i-inv BnARF2i-pFGC5941 is transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 6

Construction and transformation of RNAi cassettes that decrease *MNT* expression primarily in the integuments/seed coat of *Arabidopsis thaliana*.

This is specifically to phenocopy the big seed effect of *mnt* mutations without other effects on plant growth, development, or fertility.

The cloning strategy is shown in Fig. 15.

6a Construction of RNAi vectors containing an integument/seed coat promoter

6a(i) TT8

An RNAi vector in which the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) drives an inverted repeat of an *MNT* nucleic acid fragment (see Example 4, above) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT8* gene using the primers TT8 EcoRI F and TT8 NcoI R which introduce an EcoRI and an NcoI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' GAATTCCCAACGGGATCATGGGATTAC 3' **TT8Fi** SEQ ID NO. 19
EcoRI

5' CCATGGCGTTCCCGGAGATACGAAAAC 3' **TT8Ri** SEQ ID NO. 20

NcoI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-*MNT*-inv *MNT*i-pFGC5941 (Example 4, above), forming the vector TT8-*MNT*-inv *MNT*i-pFGC5941.

6a(ii) INO

An RNAi vector in which the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) drives an inverted repeat of an *MNT* nucleic acid fragment (see Example 4, above) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers FINOi and RINOi which introduce an EcoRI and an NcoI site at the 5' and 3' ends of the *INO* PCR fragment respectively.

5' GAATTCCCTGGATTAGTGCAAGCC 3' **FINOi** SEQ ID NO. 21
EcoRI

5' CCATGGGAGAGTGTGTGTGTACGATG 3' **RINOi** SEQ ID NO. 22
NcoI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-MNT-inv MNTi-pFGC5941 (Example 4, above), forming the vector INO-MNT-inv MNTi-pFGC5941.

6b Transformation into Arabidopsis thaliana

The TT8-MNT-inv MNTi-pFGC5941 and INO-MNT-inv MNTi-pFGC5941 vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 7

Construction and transformation of RNAi cassettes that decrease BnARF2 expression primarily in the integuments/seed coat of *Brassica napus*.

The cloning strategy is shown in Fig. 16.

7a Construction of RNAi vectors containing an integument/seed coat promoter

7a(i) TT8

An RNAi vector in which the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) drives an inverted repeat of a *BnARF2* nucleic acid fragment (see Example 5, above) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with EcoRI and NcoI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 6a(i) above.

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-BnARF2-inv BnARF2i-pFGC5941 (Example 5, above), forming the vector TT8-BnARF2-inv BnARF2i-pFGC5941.

7a(ii) INO

An RNAi vector in which the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) drives an inverted repeat of a *BnARF2* nucleic acid fragment (see Example 5, above) is constructed as described below. A 1.5 kb fragment including the *INO* promoter with EcoRI and NcoI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 6a(ii) above.

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-BnARF2-inv BnARF2i-pFGC5941 (Example 5, above), forming the vector INO-BnARF2-inv BnARF2i-pFGC5941.

7b Transformation into Brassica napus

The TT8-BnARF2-inv BnARF2i-pFGC5941 and INO-BnARF2-inv BnARF2i-pFGC5941 vectors are transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 8

Construction and transformation of an expression vector that increases *MNT* expression in *Arabidopsis thaliana*, including increased expression in the integuments/seed coat.

This is to produce a plant with altered seed size.

The cloning strategy is shown in Fig. 17.

8a Construction of a vector for constitutive expression of MNT

Construction of an expression vector with the CaMV *35S* promoter driving the *MNT* gene is described below. The *MNT* cDNA including the translational start and stop is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers 35S Xho new and 35S Bam new which introduce a XhoI and a BamHI site at the 5' and 3' ends of the *MNT* PCR fragment respectively.

5' CTCGAGGAAGGTATGGCGAGT 3' **35S Xho new** SEQ ID NO. 23
XhoI

5' GGATCCTCCAGTCTCCACCAA 3' **35S Bam new** SEQ ID NO. 24
BamHI

The *MNT* PCR fragment is A-tailed and ligated into pGEMT, and then excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of pART7, 3' to the *35S* promoter and 5' to the *ocs* terminator, forming the vector 35S-MNT-pART7.

8b Construction of binary vectors and transformation into Arabidopsis thaliana

The 35S::*MNT* expression cassette (including the *ocs* terminator signal) is excised from 35S-MNT-pART7 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector 35S-MNT-BJ40. The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

8c Analysis of seed weights in plants transformed with the 35S::MNT *cassette*
Wild-type plants transformed with the *35S::MNT* cassette described in Example 8a, b have the *mnt* mutant phenotype, including closed flowers for most of the plant's life cycle (Fig. 17B, top), and large seeds. Seeds from three independently transformed lines, along with wild-type plants grown under the same conditions, are shown in Fig. 17B, middle. The overall mean weight for these three lines was 25.5 μg, compared with 15.0 μg for the wild-type control. Expression of *MNT/ARF2* was assayed in transformed and wild-type plants by semiquantitative RT-PCR (Fig. 17B, bottom) using multiplex RT-PCR with primers RTARF2-F (5'- GAGTGGGTGGAGTGTGTTTG-3') and RTARF2-R (5'- AGTTGGTTTTCGTTTGAGCAT-3'), and control primers to the GAPC gene, GAPC-F (5'- CACTTGAAGGGTGGTGCCAAG-3') and GAPC-R (5'- CCTGTTGTCGCCAACGAAGTC-3'). PCR was initiated with RTARF2 primers and run for 4 cycles at an annealing temperature of 55°C, extension time 2 min. GAPC primers were added to each reaction mix and the reaction was run for an additional 22 cycles. This showed that plants transformed with the *35S::MNT* cassette did not have lower levels of *MNT* expression than wild-type plants; therefore the mutant phenotype was not due to cosuppression. Therefore constitutive expression of the *MNT* gene (such as achieved under control of the *35S* promoter) provides a further method for producing large seeds.

Example 9

Construction and transformation of an expression cassette that increases *BnARF2* expression in *Brassica napus*, including increased expression in the integuments/seed coat.

This is also to produce a plant with altered seed size.

The cloning strategy is shown in Fig. 17.

9a Construction of a vector for constitutive expression of BnARF2
Construction of an expression vector with the CaMV *35S* promoter driving the *BnARF2* gene is described below. The *BnARF2* cDNA from translational start to stop is amplified by

PCR from *Brassica napus* cDNA using the primers BnARF2 XhoI F and BnARF2 BamHI R which introduce a XhoI and a BamHI site at the 5' and 3' ends of the *BnARF2* PCR fragment respectively.

5' CTCGAGATGGCGAGTTCGGAGGTTTC 3' **BnARF2 XhoI F** SEQ ID NO. 25
XhoI

5' GGATCCTTAAGAGTTTCCGGCGCTGG 3' **BnARF2 BamHI R** SEQ ID NO. 26
BamHI

The *BnARF2* PCR fragment is A-tailed and ligated into pGEMT, and then excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of pART7, 3' to the *35S* promoter and 5' to the *ocs* terminator, forming the vector 35S-BnARF2-pART7.

9b Construction of binary vectors and transformation into Brassica napus

The *35S::BnARF2* expression cassette (including the *ocs* terminator signal) is excised from 35S-BnARF2-pART7 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector 35S-BnARF2-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*. Constitutive expression of the *BnARF2* gene (such as achieved under control of the *35S* promoter) provides a further method for producing large seeds.

Example 10

Construction and transformation of expression cassettes that increase *MNT* expression primarily in the integuments/seed coat of *Arabidopsis thaliana*.

The cloning strategy is shown in Fig. 18.

10a Construction of expression vectors containing an integument/seed coat promoter

10a(i) *TT8*

An expression vector based on the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(i), above. The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8-BJ36.

10a(ii) *INO*

An expression vector based on the promoter of the *INO* gene (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers INOF and INOR which introduce an NdeI and a PstI site at the 5' and 3' ends of the INO PCR fragment respectively.

5' CATATGCCTGGATTAGTGCAAGGCAA 3' **INOF** SEQ ID NO. 27
NdeI

5' CTGCAGGAGAGTGTGTGTGTACGATG 3' **INOR** SEQ ID NO. 28
PstI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO-BJ36.

10b Construction of expression vectors containing a promoter::MNT expression cassette

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above.

10b(i) *TT8-MNT*

The *MNT* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the TT8-BJ36 vector, 3' to the *TT8* promoter, forming the vector TT8-MNT-BJ36.

10b(ii) *INO-MNT*

The *MNT* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the INO-BJ36 vector, 3' to the *INO* promoter, forming the vector INO-MNT-BJ36.

10c Construction of binary vectors and transformation

10c(i) *TT8-MNT*

The *TT8::MNT* expression cassette (including the *ocs* terminator signal) is excised from TT8-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector TT8-MNT-BJ40.

10c(ii) *INO-MNT*

The *INO::MNT* expression cassette (including the *ocs* terminator signal) is excised from INO-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector INO-MNT-BJ40.

The TT8-MNT-BJ40 and INO-MNT-BJ40 binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 11

Construction and transformation of expression vectors that increase *BnARF2* expression primarily in the integuments/seed coat of *Brassica napus*.

The cloning strategy is shown in Fig. 19.

11a Construction of expression vectors containing an integument/seed coat promoter

11a(i) *TT8*

An expression vector based on the promoter of the *TT8* gene (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to

translational start of the *TT8* gene using the primers TT8F and TT8 MluI R which introduce an NdeI and an MluI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' AAACATATGCCAACGGGATCATGGGATTAC 3' **TT8F** SEQ ID NO. 11
NdeI

5' AAAACGCGTCGTTCCCGGAGATACGAAAAC 3' **TT8 MluI R** SEQ ID NO. 29
MluI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and MluI and ligated into the NdeI and MluI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8 (NdeI MluI)-BJ36.

11a(ii) *INO*

An expression vector based on the promoter of the *INO* gene (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers INOF and INO MluI R which introduce an NdeI and an MluI site at the 5' and 3' ends of the *INO* PCR fragment respectively.

5' CATATGCCTGGATTAGTGCAAGGCAA 3' **INOF** SEQ ID NO. 27
NdeI

5' ACGCGTGAGAGTGTGTGTCTACGATG 3' **INO MluI R** SEQ ID NO. 30
MluI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and MluI and ligated into the NdeI and MluI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO (NdeI MluI)-BJ36.

11b Construction of expression vectors containing a promoter::BnARF2 expression cassette

The *BnARF2* cDNA with XhoI and BamHI linkers is amplified by PCR from *Brassica napus* cDNA and ligated into pGEMT as described in Example 9a, above.

11b(i) *TT8*

The *BnARF2* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the TT8 (NdeI MluI)-BJ36 vector, 3' to the *TT8* promoter, forming the vector TT8-BnARF2-BJ36.

11b(ii) INO

The *BnARF2* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the INO (NdeI MluI)-BJ36 vector, 3' to the *INO* promoter, forming the vector INO-BnARF2-BJ36.

11c Construction of binary vectors and transformation

11c(i) *TT8*

The TT8-BnARF2 expression cassette (including the *ocs* terminator signal) is excised from TT8-BnARF2-BJ36 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector TT8-BnARF2-BJ40.

11c(ii) INO

The INO-BnARF2 expression cassette (including the *ocs* terminator signal) is excised from INO-BnARF2-BJ36 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector INO-BnARF2-BJ40.

The binary vectors TT8-BnARF2-BJ40 and INO-BnARF2-BJ40 are transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 12

Construction, transformation, and analysis of expression vectors that increase expression of a gene promoting cell division in the integuments/seed coat of *Arabidopsis thaliana*

The cloning strategy is shown in Fig. 20.

12a Construction of expression vectors containing an integument/seed coat promoter

12a(i) *TT8*

An expression vector based on the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(i), above. The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8-BJ36.

12a(ii) *TT12*

An expression vector based on the *TT12* promoter (Debeaujon *et al.*, 2000; At3g59030, accession no. AJ294464) is constructed as described below. A 1.7 kb fragment including the *TT12* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(ii), above. The *TT12* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT12-BJ36.

12a(iii) *INO*

An expression vector based on the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 10a(ii), above. The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO-BJ36.

12a(iv) BAN

An expression vector based on the promoter of the *BAN* gene (Devic *et al.*, 1999; At1g61720, accession no. AF092912) is constructed as described below. A 0.4 kb fragment including the *BAN* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *BAN* gene using the primers BANF and BANR which introduce an NdeI and a PstI site at the 5' and 3' ends of the *BAN* PCR fragment

respectively.

5' CATATGGAGAATTTGACAGATTGGTG 3' **BANF** SEQ ID NO. 31
NdeI

5' CTGCAGGTTTATCGTCTTGAGACTTC 3' **BANR** SEQ ID NO. 32
PstI

The *BAN* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector BAN-BJ36.

12b Construction of expression cassettes containing an integument/seed coat promoter driving a gene promoting cell division

12b(i) Promoter::*CYCD3;1*

Construction of expression vectors with an integument/seed coat promoter driving the *CYCD3;1* gene is described below. The *CYCD3;1* cDNA (formerly *Cycδ3*; Soni *et al.*, 1995; Vandepoele *et al.*, 2002; At4g34160, accession no. X83371) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers CYCD3F and CYCD3R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *CYCD3;1* PCR fragment respectively.

5' AAACCCGGGATGGCGATTCGGAAGGAGGAA 3' **CYCD3F** SEQ ID NO. 33
SmaI

5' AAAGGATCCTTATGGAGTGGCTACGATTGC 3 ' **CYCD3R** SEQ ID NO. 34
BamHI

The *CYCD3;1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-CYCD3;1-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-CYCD3;1-BJ36

INO-*BJ36* vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-CYCD3;1-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-CYCD3;1-BJ36

12b(ii) Promoter::*IPT1*

Construction of expression vectors with an integument/seed coat promoter driving the *IPT1* gene is described below. The *IPT1* gene (Takei *et al.*, 2001; At1g68460, accession no. AB062607) is amplified by PCR from *Arabidopsis thaliana* genomic DNA (the *IPT1* gene contains no introns) using the primers IPT1F and IPT1R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *IPT1* PCR fragment respectively.

5' AAACCCGGGATGACAGAACTCAACTTCCAC 3' **IPT1F** SEQ ID NO. 35
SmaI

5' AAAGGATCCCTAATTTTGCACCAAATGCCG 3' **IPT1R** SEQ ID NO. 36
BamHI

The *IPT1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-IPT1-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-IPT1-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-IPT1-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-IPT1-BJ36

12b(iii) Promoter::*ANT*

Construction of expression vectors with an integument/seed coat promoter driving the *ANT* gene is described below. The *ANT* gene (Klucher *et al.*, 1996; At4g37750, accession no. NM_119937) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers ANTF and ANTR which introduce a SmaI and a BamHI site at the 5' and 3' ends of the

ANT PCR fragment respectively.

5' CCCGGGGGTGTGTTCGTTGTGTAACC 3' **ANTF** SEQ ID NO. 37
SmaI

5' GGATCCGATCAAGAATCAGCCCAAGC 3' **ANTR** SEQ ID NO. 38
BamHI

The *ANT* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-ANT-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-ANT-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-ANT-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-ANT-BJ36

12b(iv) Promoter::CYCB1;1

Construction of expression vectors with an integument/seed coat promoter driving the *CYCB1;1* gene is described below. The *CYCB1;1* gene (formerly *Cyc1aAt*; Ferreira *et al.*, 1994; Vandepoele *et al.*, 2002; At4g37490, accession no. NM_119913) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers CYCB1F and CYCB1R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *CYCB1;1* PCR fragment respectively.

5' CCCGGGCACTAAGATGATGACTTCTC 3' **CB1F** SEQ ID NO. 39
SmaI

5' GGATCCAAGCGACTCATTAGACTTGT 3' **CB1R** SEQ ID NO. 40
BamHI

The *CYCB1;1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:

TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-CYCB1;1-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-CYCB1;1-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-CYCB1;1-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-CYCB1;1-BJ36

12c Construction of binary vectors and transformation into Arabidopsis thaliana

Expression cassettes (including the ocs terminator) are excised with NotI from the following vectors

TT8-CYCD3;1-BJ36
TT8-IPT1-BJ36
TT8-ANT-BJ36
TT8-CYCB1;1-BJ36
TT12-CYCD3;1-BJ36
TT12-IPT1-BJ36
TT12-ANT-BJ36
TT12-CYCB1;1-BJ36
INO-CYCD3;1-BJ36
INO-IPT1-BJ36
INO-ANT-BJ36
INO-CYCB1;1-BJ36
BAN-CYCD3;1-BJ36
BAN-IPT1-BJ36
BAN-ANT-BJ36
BAN-CYCB1;1-BJ36

and ligated into the NotI sites of the binary vector BJ40, forming the following vectors for transformation:

TT8-CYCD3;1-BJ40
TT8-IPT1-BJ40
TT8-ANT-BJ40
TT8-CYCB1;1-BJ40
TT12-CYCD3;1-BJ40
TT12-IPT1-BJ40
TT12-ANT-BJ40
TT12-CYCB1;1-BJ40
INO-CYCD3;1-BJ40
INO-IPT1-BJ40
INO-ANT-BJ40
INO-CYCB1;1-BJ40
BAN-CYCD3;1-BJ40
BAN-IPT1-BJ40
BAN-ANT-BJ40
BAN-CYCB1;1-BJ40

The binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

12d Analysis of seed weights in transformants

Results from some primary transformants using the *TT8* promoter are shown in Table 2A and Fig. 21A. The histogram shows that seeds from *TT8::CYCD3;1* and *TT8::IPT1* plants have a broader distribution and higher peak of weights than the controls. TT8::uidA lines were used as controls, as expression of the *uidA* gene is not found to affect plant growth and development. Individual *TT8::CYCD3;1* plants produced seeds up to 97% heavier than controls, with a mean increase over 27 lines of 37%. *TT8::IPT1* plants produced seeds up to 107% heavier, with a mean increase over 24 lines of 28%. The mean weights of *TT8::CYCD3;1* and *TT8::IPT1* seeds were compared with the controls using t-tests and found to be significantly different from the controls with $P<0.000$. It should be noted that some of the *TT8::IPT1* lines, including the highest weighing line, also had a vegetative phenotype including dwarfing, serrated leaves, and extremely low fertility, most likely due

to the *TT8* promoter driving vegetative expression of *IPT1* in some lines. However lines with normal vegetative development also produced large seeds. It is likely that vegetative expression of *TT8* could be prevented if required by the technique of promoter dissection (e.g. Chandrasekharan *et al.*, 2003).

	TT8::GUS (controls)	*TT8::CYCD3;1*	*TT8::IPT1*
	17.0 µg (n=52)	19.0 µg (n=77)	21.8 µg (n=45)
	14.2 (79)	21.6 (61)	15.6 (62)
	14.7 (51)	22.5 (52)	23.4 (68)
	13.9 (47)	17.9 (52)	20.9 (95)
	14.7 (66)	17.5 (63)	11.2 (58)
	15.1 (43)	16.9 (89)	19.3 (89)
	14.2 (50)	16.6 (54)	*19.9 (119)
	14.8 (61)	18.6 (57)	17.9 (64)
		13.9 (54)	17.7 (56)
		24.3 (64)	18.6 (54)
		21.9 (57)	13.2 (48)
		29.2 (49)	17.6 (72)
		18.2 (49)	20.8 (66)
		17.0 (72)	*30.6 (43)
		23.0 (45)	14.0 (61)
		18.6 (56)	19.5 (64)
		28.1 (56)	20.5 (47)
		20.1 (47)	18.8 (68)
		17.8 (47)	15.8 (65)
		18.8 (58)	22.1 (47)
		26.1 (55)	14.2 (47)
		16.4 (55)	19.6 (49)
		24.6 (56)	17.3 (50)
		19.5 (49)	*26.7 (51)
		16.1 (51)	
		25.8 (58)	
		18.0 (62)	
			*plants with very low fertility
Mean	14.8	20.3	19.0
Range	13.9 to 17.0	13.9 to 29.2	11.2 to 30.6
Standard error	0.3	0.8	0.9

Table 2A Seed weights in individual primary transformants from TT8::GUS (control), TT8::CYCD3;1, and TT8::IPT1 families

ttest for control vs TT8::CYCD3;1 and TT8:IPT1, P < 0.000, significant

Further results from plants transformed with expression vectors to increase seed size are shown in Table 2B and Fig. 21B. For these experiments, we selected kanamycin resistant lines with heavy seeds and confirmed the presence of the expression vector using PCR. For two of the lines below we weighed seeds produced by T3 plants, confirming the heritability of the large seed trait. In Table 2B, weights of controls (in this case the controls were wild-type Col-0 are shown alongside transformants where the controls and transformants were grown together. *BAN::CYCD3;1* seeds were 35% heavier than controls grown under the same conditions, and *INO::ANT* seeds were 53% heavier. *INO::ANT* seeds were also misshapen (Fig. 21B), suggesting that the expression cassette indeed affects seed coat development.

Table 2B Seed weights from plants transformed with expression cassettes to increase seed size

	transformant	w.t. Col-3 (controls)
BAN::CYCD3;1 (seeds from T2 plants)	23.9 µg	17.7 µg
INO::ANT (T2)	23.1	15.1
INO::IPT1 (T3)	26.4	
TT8::CYCD3;1 (T3)	23.2	

Example 13

Construction, transformation, and analysis of expression vectors that increase expression of a gene promoting cell division in the integuments/seed coat of *Brassica napus*

The binary vectors described in Example 12c (above) are transformed into *Brassica napus*.

Example 14

Construction of an expression vector containing a petal-and stamen-specific promoter driving *MNT* and transformation into *mnt* mutants

The cloning strategy is shown in Fig. 22.

14a Construction of an expression vector based on the AP3 promoter

An expression vector based on the promoter of the *AP3* gene (Jack *et al.*, 1992; At3g54340, accession no.AY142590) is constructed as described below. A 1 kb fragment including the *AP3* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *AP3* gene using the primers AP3F and AP3R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *AP3* PCR fragment respectively.

5' AAACATATGGATACACAAGTTCTTTGG 3' **AP3F** SEQ ID NO. 41
NdeI

5' AAACTGCAGATTCTTCTCTCTTTGTTTAA 3' **AP3R** SEQ ID NO. 42
PstI

The *AP3* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of the BJ36 vector, 5' to the *ocs* terminator signal, forming the vector AP3-BJ36.

14b Construction of an expression vector containing an AP3::MNT expression cassette

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above. The *MNT* PCR fragment is excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of the AP3-BJ36 vector, 3' to the AP3 promoter and 5' to the *ocs* terminator, forming the vector AP3-MNT-BJ36.

14c Construction of binary vector and transformation into Arabidopsis thaliana
The *AP3::MNT* expression cassette (including the *ocs* terminator signal) is excised from AP3-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector AP3-MNT-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 15

Construction of an expression vector containing a sepal-and petal-specific promoter driving *MNT* and transformation into *mnt* mutants

The cloning strategy is shown in Fig. 23.

15a Construction of an expression vector based on the AP1 promoter

An expression vector based on the promoter of the *AP1* gene (Mandel *et al.*, 1992; At1g69120, accession no. NM_105581) is constructed as described below. A 1.7 kb fragment including the *AP1* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *AP1* gene using the primers AP1F and AP1R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *AP3* PCR fragment respectively.

5' CATATG GTGACATCTTTTTAGCATAGGTTC 3' **AP1F** SEQ ID NO. 43
NdeI

5' CTGCAG TTTTGATCCTTTTTTAAGAAACTT 3' **AP1R** SEQ ID NO. 44
PstI

The *AP1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of the BJ36 vector, 5' to the *ocs* terminator signal, forming the vector AP1-BJ36.

15*b Construction of an expression vector containing an AP1::MNT expression cassette*

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above. The *MNT* PCR fragment is excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of the AP1-BJ36 vector, 3' to the AP1 promoter and 5' to the *ocs* terminator, forming the vector AP1-MNT-BJ36.

15c Construction of binary vector and transformation into Arabidopsis thaliana
The *AP1::MNT* expression cassette (including the *ocs* terminator signal) is excised from AP1-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector AP1-MNT-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana*.

Example 16

Value of *mnt* mutants in understanding and modifying growth of the inflorescence stem

mnt-1 mutants have thick inflorescence stems compared with wild-type plants. The increased diameter of *mnt-1* stems is caused by extra cell divisions (Fig. 24B). Therefore it is expected that stem thickness may be increased in other species by altering expression of an *MNT* orthologue and thereby increasing the number of cells in the stem.

Example 17

Construction and transformation of an RNAi cassette that decreases *MNT* expression in *Arabidopsis thaliana*, including decreased expression in the stem.

The cloning and transformation strategy is described in Example 4. The cloning strategy is shown in Fig. 13A. Transformed plants have an increased stem diameter with respect to wild type (mean inflorescence stem diameter between nodes 2 and 3: w.t., 1.293 ± s.e.m. 0.4 mm, n=13; *35S::MNT* RNAi, 1.419 ± 0.4, n=14; two-tailed t-test shows that diameters of w.t. and *35S::MNT* RNAi stems are significantly different at $P<0.05$). The stem phenotype of transformed plants compared with wild-type plants is shown in Fig. 13B.

Example 18

Construction of an expression vector containing a flower-preferred promoter driving *MNT* and transformation into *mnt* mutants

The cloning strategy is shown in Fig. 25.

18a Construction of an expression vector based on the LFY promoter

An expression vector based on the promoter of the *LFY* gene (Weigel *et al.*, 1992; At5g61850, accession no. NM_125579) is constructed as described below. A 2.1 kb fragment including the *LFY* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *LFY* gene using the primers LFYF and LFYR which introduce an NdeI and a PstI site at the 5' and 3' ends of the *AP3* PCR fragment respectively.

5' CATATG TGTAACTGCAAAGTGTAGTTCGG 3' **LFYF** SEQ ID NO. 45
NdeI

5' CTGCAG AATCTATTTTTCTCTCTCTCTC 3' **LFYR** SEQ ID NO. 46
PstI

The *LFY* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of the BJ36 vector, 5' to the *ocs* terminator signal, forming the vector LFY-BJ36.

18*b Construction of an expression vector containing an LFY::MNT expression cassette*

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above. The *MNT* PCR fragment is excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of the LFY-BJ36 vector, 3' to the LFY promoter and 5' to the *ocs* terminator, forming the vector AP1-LFY-BJ36.

18c Construction of binary vector and transformation into Arabidopsis thaliana
The *LFY::MNT* expression cassette (including the *ocs* terminator signal) is excised from AP1-LFY-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector AP1-LFY-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

SEQ ID NOS

1 *MNT* genomic DNA w.t Col-0
2 *MNT* cDNA w.t.Col-0
3 MNT predicted protein w.t. Col-0
4 *mnt-1* genomic DNA Col-3
5 *mnt-1* cDNA Col-3, translational start to stop
6 mnt-1 predicted protein Col-3
7 F primer for amplifying *Brassica napus ARF2* cDNA

8 R primer for amplifying *Brassica napus ARF2* cDNA
9 *BnARF2* cDNA, translational start to stop
10 *BnARF2* predicted protein
11 F primer for *TT8* promoter with NdeI linker
12 R primer for *TT8* promoter with PstI linker
13 F primer for *TT12* promoter with NdeI linker
14 R primer for *TT12* promoter with PstI linker
15 F primer for *MNT* RNAi fragment with XbaI and AscI linkers
16 R primer for *MNT* RNAi fragment with BamHI and SawI linkers
17 F primer for *BnARF2* RNAi fragment with XbaI and AscI linkers
18 R primer for *BnARF2* RNAi fragment with BamHI and SwaI linkers
19 F primer for *TT8* promoter with EcoRI linker
20 R primer for *TT8* promoter with NcoI linker
21 F primer for *INO* promoter with EcoRI linker
22 R primer for *INO* promoter with NcoI linker
23 F primer for *MNT* cDNA with XhoI linker
24 R primer for *MNT* cDNA with BamHI linker
25 F primer for *BnARF2* cDNA with XhoI linker
26 R primer for *BnARF2* cDNA with BamHI linker
27 F primer for *INO* promoter with NdeI linker
28 R primer for *INO* promoter with PstI linker
29 R primer for *TT8* promoter with MluI linker
30 R primer for *INO* promoter with MluI linker
31 F primer for *BAN* promoter with NdeI linker
32 R primer for *BAN* promoter with PstI linker
33 F primer for *CYCD3;1* cDNA with SmaI linker
34 R primer for *CYCD3;1* cDNA with BamHI linker
35 F primer for *IPT1* cDNA with SmaI linker
36 R primer for *IPT1* cDNA with BamHI linker
37 F primer for *ANT* cDNA with SmaI linker
38 R primer for *ANT* cDNA with BamHI linker
39 F primer for *CYCB1;1* cDNA with SmaI linker
40 R primer for *CYCB1;1* cDNA with BamHI linker

41 F primer for *AP3* promoter with NdeI linker

42 R primer for *AP3* promoter with PstI linker

43 F primer for *AP1* promoter with NdeI linker

44 R primer for *AP1* promoter with PstI linker

45 F primer for *LFY* promoter with NdeI linker

46 R primer for *LFY* promoter with PstI linker

SEQ ID NO. 1

MNT wild-type genomic DNA, Col-0

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SEQ ID NO. 2

MNT complete cDNA wild-type Col-0

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SEQ ID NO. 3

MNT predicted protein wild-type Col-0

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SEQ ID NO. 4

mnt-1 genomic DNA Col-3

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SEQ ID NO. 5

mnt-1 cDNA translational start to stop

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SEQ ID NO. 6

mnt-1 predicted protein Col-3

MASSEVSMKGNRGGDNFSSSGFSDPKETRNVSVAGEGQKSNSTRSAAAER
ALDPEAALYRELWHACAGPLVTVPRQDDRVFYFPQGHIEQVEASTNQAAE
QQMPLYDLPSKLLCRVINVDLKRQIQMKFMRRLLFFLRLIKTRMQLRKKRL
FLHLRGSRCIRSAKP

SEQ ID NO. 7

Forward primer for amplifying *Brassica napus ARF2*

5' ATGGCGAGTTCGGAGGTTT 3'

SEQ ID NO. 8

Reverse primer for amplifying *Brassica napus ARF2*

5' TGGACAATGAAGGATTTGATG 3'

SEQ ID NO. 9

BnARF2 cDNA, translational start to stop

ATGGCGAGTTCGGAGGTTTCTATGAAAGGAAATCGTGGACGAGGAGAA
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AAGTCTGCATCAAATCCTTCATTGTCCAGCGCCGGAAACTCTTAA

SEQ ID NO. 10

BnARF2 predicted protein

MASSEVSMKGNRGRGENFSSAGYSDPTVAGEAQKTQSNRSVAAERVVDPE
AALYRELWHACAGPLVTVPRQDDRVFYFPQGHIEQVEASTNQAAEQQMPL

YDLPSKILCRVINVDLKAEADTDEVYAQITLLPEPVQDENSIEKEAPPPPPPR
FQVHSFCKTLTASDTSTHGGFSVLRRHADECLPPLDMSRQPPTQELVAKDL
HASEWRFRHIFRGQPRRHLLQSGWSVFVSSKRLVAGDAFIFLRGENGELRV
GVRRAMRQQGNVPSSVISSHSMHLGVLATAWHAISTGTMFTVYYKPRTSP
SEFIVPFDQYTESVKINYSIGMRFKMRFEGEEAPEQRFTGTIVGIEDSDPTRW
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KDWLIVYTDDENDMMLVGDDPWQEFCCMVRKIFIYTKEEVRKMNPGTLC
CRNEEEPVVGEGSDAKDAKSASNPSLSSAGNS

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